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| (54) Title: POLYPHOSPHAZENES USEFUL AS IMMUNOADJUVANTS (57) Abstract An immunoadjuvant soluble phosphazene polyelectrolyte is disclosed. In one embodiment, the polymeric adjuvant is a poly(organophosphazene) with (i) ionized or ionizable pendant groups that contain, for example, carboxylic acid, sulfonic acid, or hydroxyl moieties, and (ii) pendant groups that are susceptible to hydrolysis under the conditions of use, to impart biodegradability to the polymer. | | |

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POLYPHOSPHAZENES USEFUL AS IMMUNOADJUVANTS

Background of the Invention

This application is in the area of polymers for biomedical applications, and in particular describes polymers that can be used as immunoadjuvants.

Vaccine Development

A wide variety of antigens stimulate the production of antibodies in animals and confer protection against subsequent infection. However, some antigens are unable to stimulate an effective immune response.

The immunogenicity of a relatively weak antigen is often enhanced by the simultaneous administration of the antigen with an adjuvant, a substance that is not immunogenic when administered alone, but will induce a state of mucosal and/or systemic immunity when combined with the antigen. It has been traditionally thought that adjuvants, such as mineral oil emulsions or aluminum hydroxide, form an antigen depot at the site of injection that slowly releases antigen. Recent studies by Allison and Byars, in: "Vaccines: New Approaches to Immunological Problems", R.W. Ellis, ed., p. 431, Butterworth-Heinemann, Oxford (1992) indicate that adjuvants enhance the immune response by stimulating specific and sometimes very narrow branches of the immune response by the release of cytokines. Unfortunately, many immunoadjuvants, such as Freund's Complete Adjuvant, are toxic and are therefore only useful for animal research purposes, not human vaccinations. Freund's Complete Adjuvant contains a suspension of heat-killed *Mycobacterium tuberculosis* in mineral oil containing a surfactant and causes granulomatous lesions in animals at the site of immunization.

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Freund's adjuvant may also cause the recipient of a vaccine to test positive for tuberculosis.

Some synthetic polyelectrolytes have been found to provide immunostimulation when combined with an antigen. For example, the adjuvant activity of polyacrylic acid (PAA), copolymers of acrylic acid and N-vinylpyrrolidone (CP-AA-VPD), poly-2-methyl-5-vinyl pyridine (PMVP), poly-4-vinyl-N-ethylpyridinium bromide (PVP-R₂) and similar compounds, when conjugated to an antigen, has been studied by Petrov et. al., *Jhurnal Vses. Khim. Obva im. D.I.Mendeleeva*, 33:22-42 (1988). The immunomodulatory effect of polyelectrolyte complexes containing many of these same polyelectrolytes has also been more recently reviewed by Petrov, et al., *Sov. Med. Rev. D. Immunol.*, 4:1-113 (1992). However, the toxicity and biodegradability of these polymers has not been studied and may prevent use of these polymers as adjuvants for use in humans.

A non-toxic adjuvant or carrier having the ability to stimulate an immune response to non-antigenic or weakly antigenic molecules would be useful in the development and administration of vaccines.

Therefore, it is an object of the present invention to provide an adjuvant that can be safely administered to humans and animals with minimal toxicity.

It is a further object of the present invention to provide an adjuvant that is soluble and biodegradable.

It is a further object of the present invention to provide a vaccine that confers protection against an organism such as the influenza virus or *Clostridium tetani* bacteria.

It is a further object of the present

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invention to provide a rapid and efficient method of synthesizing a polymer, such as polyphosphazene, for use as an adjuvant.

Summary of the Invention

5 A synthetic, water-soluble polyphosphazene is disclosed for use as an adjuvant. In a preferred embodiment, the phosphazene is a polyelectrolyte that is biodegradable and that exhibits minimal toxicity when administered to animals, such as
10 humans.

In one embodiment, the polymeric adjuvant is an poly(organophosphazene) with (i) ionized or ionizable pendant groups that contain, for example, carboxylic acid, sulfonic acid, or hydroxyl
15 moieties, and (ii) pendant groups that are susceptible to hydrolysis under the conditions of use, to impart biodegradability to the polymer. Suitable hydrolyzable groups include, for example, chlorine, amino acid, amino acid ester, imidazole,
20 glycerol, and glucosyl.

Two examples of polyphosphazenes that are useful as immunoadjuvants are poly[di(carboxylatophenoxy)-phosphazene-co-di(glycinato)phosphazene-co-
25 (carboxylatophenoxy)(glycinato)phosphazene] and poly[di(carboxylatophenoxy)phosphazene-co-di(chloro)phosphazene-co-(carboxylatophenoxy)-(chloro)phosphazene].

A vaccine composition is prepared by either
30 mixing or conjugating the polymer adjuvant with an antigen prior to administration. Alternatively, the polymer and antigen can be administered separately to the same site.

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When cross-linked with a multivalent ion, the polymer becomes less soluble, resulting in slower release of the polymer from the site of administration.

5 Detailed Description of the Invention

The term amino acid, as used herein, refers to both natural and synthetic amino acids, and includes, but is not limited to alanyl, valinyl, leucinyl, isoleucinyl, prolinyl, phenylalaninyl, 10 tryptophanyl, methioninyl, glycynyl, serinyl, threoninyl, cysteinyl, tyrosinyl, asparaginyl, glutaminyl, aspartoyl, glutaoyl, lysinyl, argininyl, and histidinyl.

The term amino acid ester refers to the 15 aliphatic, aryl or heteroaromatic carboxylic acid ester of a natural or synthetic amino acid.

The term alkyl, as used herein, refers to a saturated straight, branched, or cyclic hydrocarbon, or a combination thereof, typically of 20 C_1 to C_{20} , and specifically includes methyl, ethyl, propyl, isopropyl, butyl, isobutyl, t-butyl, pentyl, cyclopentyl, isopentyl, neopentyl, hexyl, isohexyl, cyclohexyl, 3-methylpentyl, 2,2-dimethylbutyl, 2,3-dimethylbutyl, heptyl, 25 octyl, nonyl, and decyl.

The term (alkyl or dialkyl)amino refers to an amino group that has one or two alkyl substituents, respectively.

The terms alkenyl and alkynyl, as used herein, 30 refers to a C_2 to C_{20} straight or branched hydrocarbon with at least one double or triple bond, respectively.

The term aryl, as used herein, refers to phenyl or substituted phenyl, wherein the 35 substituent is halo, alkyl, alkoxy, alkylthio,

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haloalkyl, hydroxyalkyl, alkoxyalkyl,
methylenedioxy, cyano, C(O) (lower alkyl),
-CO₂H, -SO₃H, -PO₃H, -CO₂alkyl, amide, amino,
alkylamino and dialkylamino, and wherein the aryl
5 group can have up to 3 substituents.

The term aliphatic refers to hydrocarbon,
typically of C₁ to C₂₀, that can contain one or a
combination of alkyl, alkenyl, or alkynyl moieties,
and which can be straight, branched, or cyclic, or
10 a combination thereof.

The term halo, as used herein, includes
fluoro, chloro, bromo, and iodo.

The term aralkyl refers to an aryl group with
an alkyl substituent.

15 The term alkaryl refers to an alkyl group that
has an aryl substituent, including benzyl,
substituted benzyl, phenethyl or substituted
phenethyl, wherein the substituents are as defined
above for aryl groups.

20 The term heteroaryl or heteroaromatic, as used
herein, refers to an aromatic moiety that includes
at least one sulfur, oxygen, or nitrogen in the
aromatic ring, and that can be optionally
substituted as described above for aryl groups.

25 Nonlimiting examples are furyl, pyridyl, pyrimidyl,
thienyl, isothiazolyl, imidazolyl, tetrazolyl,
pyrazinyl, benzofuranyl, benzothiophenyl, quinolyl,
isoquinolyl, benzothienyl, isobenzofuryl,
pyrazolyl, indolyl, isoindolyl, benzimidazolyl,
30 purinyl, carbozolyl, oxazolyl, thiazolyl,
isothiazolyl, 1,2,4-thiadiazolyl, isooxazolyl,
pyrrolyl, pyrazolyl, quinazolinyl, pyridazinyl,
pyrazinyl, cinnolinyl, phthalazinyl, quinoxalinyl,
xanthinyl, hypoxanthinyl, pteridinyl, 5-
35 azacytidinyl, 5-azauracilyl, triazolopyridinyl,
imidazolopyridinyl, pyrrolopyrimidinyl, and
pyrazolopyrimidinyl.

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The term "pharmaceutically acceptable cation" refers to an organic or inorganic moiety that carries a positive charge and that can be administered as a counteranion in a phosphazene polyelectrolyte.

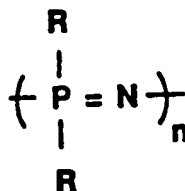
The term heteroalkyl, as used herein, refers to a alkyl group that includes a heteroatom such as oxygen, sulfur, or nitrogen (with valence completed by hydrogen or oxygen) in the carbon chain or terminating the carbon chain.

A synthetic polymer is provided for use as an immunoadjuvant. The polymer adjuvant is a polyphosphazene that is at least partially soluble in water (typically to an extent of at least 0.001% by weight), an aqueous buffered salt solution, or aqueous alcohol solution. The polyphosphazene preferably contains charged side groups, either in the form of an acid or base that is in equilibrium with its counter ion, or in the form of an ionic salt thereof.

The polymer is preferably biodegradable and exhibits minimal toxicity when administered to animals, including humans.

Selection of Polyphosphazene Polyelectrolytes.

Polyphosphazenes are polymers with backbones consisting of alternating phosphorus and nitrogen, separated by alternating single and double bonds. Each phosphorous atom is covalently bonded to two pendant groups ("R"). The repeat unit in polyphosphazenes has the following general formula:



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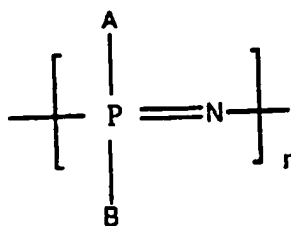
wherein n is an integer.

The substituent ("R") can be any of a wide variety of moieties that can vary within the polymer, including but not limited to aliphatic, aryl, aralkyl, alkaryl, carboxylic acid, heteroaromatic, carbohydrates, including glucose, heteroalkyl, halogen, (aliphatic)amino- including alkylamino-, heteroaralkyl, di(aliphatic)amino- including dialkylamino-, arylamino-, diarylamino-, alkylaryl amino-, -oxyaryl including but not limited to -oxyphenylCO₂H, -oxyphenylSO₃H, -oxyphenylhydroxyl and -oxyphenylPO₃H; -oxyaliphatic including -oxyalkyl, -oxy(aliphatic)CO₂H, -oxy(aliphatic)SO₃H, -oxy(aliphatic)PO₃H, and -oxy(aliphatic)hydroxyl, including -oxy(alkyl)hydroxyl; -oxyalkaryl, -oxyaralkyl, -thioaryl, -thioaliphatic including -thioalkyl, -thioalkaryl, -thioaralkyl, -NHC(O)O-(aryl or aliphatic), -O-[(CH₂)_xO]_y-CH₂)_xNH₂, -O-[(CH₂)_xO]_yCH₂)_xNH(CH₂)_xSO₃H, and -O-[(CH₂)_xO]_y-(aryl or aliphatic), wherein x is 1-8 and y is an integer of 1 to 20. The groups can be bonded to the phosphorous atom through, for example, an oxygen, sulfur, nitrogen, or carbon atom.

In general, when the polyphosphazene has more than one type of pendant group, the groups will vary randomly throughout the polymer, and the polyphosphazene is thus a random copolymer. Phosphorous can be bound to two like groups, or two different groups. Polyphosphazenes with two or more types of pendant groups can be produced by reacting poly(dichlorophosphazene) with the desired nucleophile or nucleophiles in a desired ratio. The resulting ratio of pendant groups in the polyphosphazene will be determined by a number of factors, including the ratio of starting materials

used to produce the polymer, the temperature at which the nucleophilic substitution reaction is carried out, and the solvent system used. While it is very difficult to determine the exact substitution pattern of the groups in the resulting polymer, the ratio of groups in the polymer can be easily determined by one skilled in the art.

In one embodiment, the immunoadjuvant is a biodegradable polyphosphazene of the formula:



10 wherein A and B can vary independently in the polymer, and can be:

(i) a group that is susceptible to hydrolysis under the conditions of use, including but not limited to chlorine, amino acid, amino acid ester (bound through the amino group), imidazole, glycerol, or glucosyl; or

(ii) a group that is not susceptible to hydrolysis under the conditions of use, including, but not limited to an aliphatic, aryl, aralkyl, alkaryl, carboxylic acid, heteroaromatic, heteroalkyl, (aliphatic)amino- including alkylamino-, heteroaralkyl, di(aliphatic)amino- including dialkylamino-, arylamino-, diarylamino-, alkylaryl amino-, -oxyaryl including but not limited to -oxyphenylCO₂H, -oxyphenylSO₃H, -oxyphenylhydroxyl and -oxyphenylPO₃H; -oxyaliphatic including -oxyalkyl, -oxy(aliphatic)CO₂H, -oxy(aliphatic)SO₃H, -oxy(aliphatic)PO₃H, and -oxy(aliphatic)hydroxyl, including -oxy(alkyl)hydroxyl; -oxyalkaryl,

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-oxyaralkyl, -thioaryl, -thioaliphatic including
-thioalkyl, -thioalkaryl, or thioaralkyl;

wherein the polymer contains at least one
percent or more, preferably 10 percent or more, and
5 more preferably 80 to 90 percent or more, but less
than 100%, of repeating units that are not
susceptible to hydrolysis under the conditions of
use, and

wherein n is an integer of 4 or more, and
10 preferably between 10 and 20,000.

It should be understood that certain groups,
such as heteroaromatic groups other than imidazole,
hydrolyze at an extremely slow rate under neutral
aqueous conditions, such as that found in the
15 blood, and therefore are typically considered
nonhydrolyzable groups for purposes herein.
However, under certain conditions, for example, low
pH, as found, for example, in the stomach, the rate
of hydrolysis of normally nonhydrolyzable groups
20 (such as heteroaromatics other than imidazole) can
increase to the point that the biodegradation
properties of the polymer can be affected. One of
ordinary skill in the art using well known
techniques can easily determine whether pendant
25 groups hydrolyze at a significant rate under the
conditions of use. One of ordinary skill in the
art can also determine the rate of hydrolysis of
the polyphosphazenes of diverse structures as
described herein, and will be able to select that
30 polyphosphazene that provides the desired
biodegradation profile for the targeted use.

The degree of hydrolytic degradability of the
polymer will be a function of the percentage of
pendant groups susceptible to hydrolysis and the
35 rate of hydrolysis of the hydrolyzable groups. The
hydrolyzable groups are replaced by hydroxyl groups
in aqueous environments to provide P-OH bonds that

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impart hydrolytic instability to the polymer.

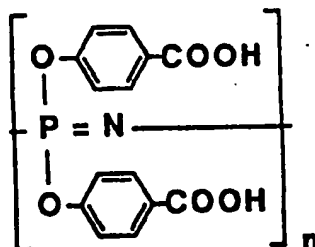
In other embodiments, the immunoadjuvant is:

- (i) a nonbiodegradable polyphosphazene wherein none, or virtually none, of the pendant groups in the polymer are susceptible to hydrolysis under the conditions of use, or (ii) a completely biodegradable polyphosphazene wherein all of the groups are susceptible to hydrolysis under the conditions of use (for example, poly[di(glycinato)-phosphazene]).

Phosphazene polyelectrolytes are defined herein as polyphosphazenes that contain ionized or ionizable pendant groups that render the polyphosphazene anionic, cationic or amphophilic. The ionic groups can be in the form of a salt, or, alternatively, an acid or base that is or can be at least partially dissociated. Any pharmaceutically acceptable monovalent cation can be used as counterion of the salt, including but not limited to sodium, potassium, and ammonium. The phosphazene polyelectrolytes can also contain non-ionic side groups. The phosphazene polyelectrolyte can be biodegradable or nonbiodegradable under the conditions of use. The ionized or ionizable pendant groups are preferably not susceptible to hydrolysis under the conditions of use.

A preferred phosphazene polyelectrolyte immunoadjuvant contains pendant groups that include carboxylic acid, sulfonic acid, or hydroxyl moieties. While the acidic groups are usually on nonhydrolyzable pendant groups, they can alternatively, or in combination, also be positioned on hydrolyzable groups. An example of a phosphazene polyelectrolyte having carboxylic acid groups as side chains is shown in the following formula:

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wherein n is an integer, preferably an integer between 10 and 10,000. This polymer has the chemical name

poly[di(carboxylatophenoxy)phosphazene] or,
 5 alternatively,
 poly[bis(carboxylatophenoxy)phosphazene] (PCPP).

The phosphazene polyelectrolyte is preferably biodegradable to prevent eventual deposition and accumulation of polymer molecules at distant sites
 10 in the body, such as the spleen. The term biodegradable, as used herein, means a polymer that degrades within a period that is acceptable in the desired application, typically less than about five years and most preferably less than about one year,
 15 once exposed to a physiological solution of pH 6-8 at a temperature of approximately 25°C - 37°C.

Most preferably the polymer is a poly(organophosphazene) that includes pendant
 20 groups that include carboxylic acid moieties that do not hydrolyze under the conditions of use and pendant groups that are susceptible to hydrolysis under the conditions of use. Examples of preferred phosphazene polyelectrolytes with
 25 hydrolysis-sensitive groups are poly[di(carboxylatophenoxy)phosphazene-co-di(amino acid)phosphazene-co-(carboxylatophenoxy)(amino acid)phosphazene], specifically including
 30 poly[di(carboxylatophenoxy)phosphazene-co-di(glycinato)phosphazene-co-

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(carboxylatophenoxy) (glycinato)phosphazene], and poly[di(carboxylatophenoxy)phosphazene-co-di(chloro)phosphazene-co-(carboxylatophenoxy) (chloro)phosphazene].

5 The toxicity of the polyphosphazene can be determined using cell culture experiments well known to those skilled in the art. For example, toxicity of poly[di(carboxylatophenoxy)phosphazene] was determined in cell culture by coating cell
10 culture dishes with the poly[di(carboxylatophenoxy)phosphazene]. Chicken embryo fibroblasts were then seeded onto the coated petri dishes. Three days after seeding the chicken embryo fibroblasts, the cells had become flattened
15 and spindles formed. Under phase contrast microscopy, mitotic figures were observed. These observations provide evidence of the non-toxicity of poly[di(carboxylatophenoxy)-phosphazene] to replicating cells.

20 Crosslinked polyphosphazenes for use as immunoadjuvants can be prepared by combining a phosphazene polyelectrolyte with a metal multivalent cation such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt,
25 nickel, or cadmium.

Synthesis of Phosphazene Polyelectrolytes

 Polyphosphazenes, including phosphazene polyelectrolytes, can be prepared by a macromolecular nucleophilic substitution reaction
30 of poly(dichlorophosphazene) with a wide range of chemical reagents or mixture of reagents in accordance with methods known to those skilled in the art. Preferably, the phosphazene polyelectrolytes are made by reacting the
35 poly(dichlorophosphazene) with an appropriate nucleophile or nucleophiles that displace chlorine. Desired proportions of hydrolyzable to non-

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hydrolyzable side chains in the polymer can be obtained by adjusting the quantity of the corresponding nucleophiles that are reacted with poly(dichlorophosphazene) and the reaction conditions as necessary. Preferred polyphosphazenes for immunoadjuvant activity have a molecular weight of over 1,000.

For example, poly[(carboxylatophenoxy)-(glycinato)phosphazene] (PC-GlPP) is prepared by the nucleophilic substitution reaction of the chlorine atoms of the poly(dichlorophosphazene) with propyl p-hydroxybenzoate and ethyl glycinate hydrochloride (PC-GlPP synthesis). The poly[(aryloxy)(glycinato)phosphazene] ester thus obtained is then hydrolyzed to the corresponding poly(carboxylic acid). Other polyphosphazenes can be prepared as described by Allcock, H.R.; et al., *Inorg. Chem.* 11, 2584 (1972); Allcock, H.R.; et al., *Macromolecules* 16, 715 (1983); Allcock, H.R.; et al., *Macromolecules* 19, 1508 (1986); Allcock, H.R.; et al., *Biomaterials* 19, 500 (1988); Allcock, H.R.; et al., *Macromolecules* 21, 1980 (1988); Allcock, H.R.; et al., *Inorg. Chem.* 21(2), 515-521 (1982); Allcock, H.R.; et al., *Macromolecules* 22:75-79 (1989); U.S. Patent Nos. 4,440,921, 4,495,174, 4,880,622 to Allcock, H.R.; et al.,; U.S. Patent No. 4,946,938 to Magill, et al., U.S. Patent No. 5,149,543 to Cohen et al., and the publication of Grolleman, et al., *J. Controlled Release* 3,143 (1986), the teachings of which, and polymers disclosed therein, are incorporated by reference herein.

Selection of an Antigen

The antigen can be derived from a cell, bacteria, or virus particle, or portion thereof. As defined herein, antigen may be a protein, peptide, polysaccharide, glycoprotein, glycolipid,

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nucleic acid, or combination thereof, which elicits an immunogenic response in an animal, for example, a mammal, bird, or fish. As defined herein, the immunogenic response can be humoral or cell-mediated. In the event the material to which the immunogenic response is to be directed is poorly antigenic, it may be conjugated to a carrier such as albumin or to a hapten, using standard covalent binding techniques, for example, with one of the several commercially available reagent kits.

In one embodiment, the polymer is used to deliver nucleic acid which encodes antigen to a mucosal surface where the nucleic acid is expressed.

Examples of preferred antigens include viral proteins such as influenza proteins, human immunodeficiency virus (HIV) proteins, and hepatitis B proteins, and bacterial proteins and lipopolysaccharides such as gram negative bacterial cell walls and *Neisseria gonorrhea* proteins.

Preparation of an Immunogenic Composition

Combining Antigen with polymer for simultaneous administration.

An immunogenic composition, or vaccine, is prepared by combining the polymer adjuvant with an antigen. Approximately 0.5 - 0.0001 parts of antigen is added to one part polymer, preferably by stirring a solution of polymer and antigen until a solution or suspension is obtained, preferably for 10 minutes or more at 25°C. The polymer is preferably combined with the antigen using a method dispersing the antigen uniformly throughout the adjuvant. Methods for liquifying the polymer include dissolving the polymer in an aqueous-based solvent, preferably having a pH range of between 7.1 and 7.7, and melting the polymer. The latter is useful only when the antigen is stable at the

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polymer melting temperature. The antigen is then mixed with the polymer. The polymer and the antigen, in solid form, for example, when the antigen is lyophilized, can also be physically mixed together, for example, by compression molding. The polymer can also be used to encapsulate the antigen, for example, using the method of U.S. Patent 5,149,543 to Cohen, et al., the teachings of which are incorporated herein, or by spray drying a solution of polymer and antigen. Alternatively, microspheres containing the antigen and adjuvant can be prepared by simply mixing the components in an aqueous solution, and then coagulating the polymer together with the substance by mechanical forces to form a microparticle. The microparticle can be stabilized, if necessary or desired, using electrolytes, pH changes, organic solvents, heat or frost to form polymer matrices encapsulating biological material.

In a preferred embodiment, approximately one part of polymer is dissolved in 10 parts 3% Na_2CO_3 aqueous solution while stirring, then 10 to 90 parts phosphate buffer pH 7.4 is slowly added.

Polymer-Antigen Conjugates

The polymer can also be covalently conjugated with the antigen to create a water-soluble conjugate in accordance with methods well-known to those skilled in the art, usually by covalent linkage between an amino or carboxyl group on the antigen and one of the ionizable side groups on the polymer.

Cross-linked Polymer Adjuvant

In an alternative preferred embodiment, the polymer is cross-linked with a multivalent ion, preferably using an aqueous solution containing multivalent ions of the opposite charge to those of the charged side groups of the polyphosphazene,

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such as multivalent cations if the polymer has acidic side groups or multivalent anions if the polymer has basic side groups.

Preferably, the polymers are cross-linked by di and trivalent metal ions such as calcium, copper, aluminum, magnesium, strontium, barium, tin, zinc, and iron, organic cations such as poly(amino acid)s, or other polymers such as poly(ethyleneimine), poly(vinylamine) and polysaccharides.

Additives to the polymer-adjuvant mixture.

It will be understood by those skilled in the art that the immunogenic vaccine composition can contain other physiologically acceptable ingredients such as water, saline or a mineral oil such as Drakeol™, Markol™, and squalene, to form an emulsion.

Administration of Polymer-Antigen Vaccine

The immunogenic composition can be administered as a vaccine by any method known to those skilled in the art that elicits an immune response, including parenterally, orally, or by transmembrane or transmucosal administration. Preferably, the vaccine is administered parenterally (intravenously, intramuscularly, subcutaneously, intraperitoneally, etc.), and preferably subcutaneously. Nonlimiting examples of routes of delivery to mucosal surfaces are intranasal (or generally, the nasal associated lymphoid tissue), respiratory, vaginal, and rectal.

The dosage is determined by the antigen loading and by standard techniques for determining dosage and schedules for administration for each antigen, based on titer of antibody elicited by the polymer-antigen administration, as demonstrated by the following examples.

Although in the preferred embodiment the

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polymer-antigen mixture is administered simultaneously, in an alternative embodiment, the polymer and antigen are administered separately to the same or nearby site. The polymer serves to
5 attract cells of the immune system to the site, where they process the antigen.

The polyphosphazene adjuvants and methods of synthesis will be further understood by reference to the following non-limiting examples.

10 **Example 1: Synthesis of
 poly[(carboxylatophenoxy)-
 (glycinato)phosphazene].**

Poly[(carboxylatophenoxy)(glycinato)phosphazene] was prepared as follows. Poly(dichlorophosphazene)
15 (5.0, 0.0425 moles) was dissolved in 300 ml tetrahydrofuran (THF). The sodium salt of propyl p-hydroxybenzoate (prepared by reacting propyl hydroxybenzoate (30.6 g, 0.17 moles) with 60% sodium hydride (6.12, 0.15 moles) in THF) was added
20 dropwise to the dissolved polymer. After addition of the sodium salt, the reaction mixture was stirred at reflux for 2 days and monitored by ³¹P NMR.

Ethyl glycinate hydrochloride (23.63 g, 0.17
25 moles) was suspended in 50 ml toluene containing triethylamine (23.69, 0.17 moles) and refluxed for 3.5 hours. The reaction mixture was cooled in an ice bath and triethylamine hydrochloride precipitated from the solution. The solution was
30 filtered and added to the polymer mixture at 0°C. The reaction mixture was allowed to warm to room temperature and stirred for 2 days. The polymer was purified by repeated precipitations into 100% ethanol.

35 The resulting polymer (0.5 g, 1.33 mmol) was dissolved in dry THF (20 ml). The solution was added slowly to a mixture of potassium tert-

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butoxide and water in dry THF. For the first 5 minutes, the mixture was cooled to 0°C; it was then stirred at room temperature for 40 hours. A large excess of ice water (300 ml) was added, and the solution was concentrated by evaporation. The polymer was isolated by acidification of the solution with hydrochloric acid to pH 5.5. The conditions of reactions and weight average molecular weights of obtained polymers measured by gel permeation chromatography in water is shown in Table 1 below.

Table 1. Synthesis of poly[(carboxylatophenoxy)(glycinato)phosphazene].

| No | Concentration of polymer % w/v mol/l | Concentration of potassium tert-butoxide mol/l | Reaction of water mol/l | MW timekDa hours |
|----|---|---|-------------------------------|------------------------|
| 1 | 0.42 | 0.30 | 0.1 | 42 80 |
| 2 | 0.42 | 0.15 | 0.05 | 18 130 |
| 3 | 0.42 | 0.04 | 0.05 | 5 170 |

The structures of polymers were confirmed by ^1H and ^{31}P NMR (JEOL FX90Q NMR spectrometer) and elemental microanalysis.

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Example 2: Synthesis of Poly[di(carboxylatophenoxy)phosphazene].

Poly[di(carboxylatophenoxy)phosphazene] was prepared by chemical modification of
5 poly(dichlorophosphazene) with the sodium salt of propyl p-hydroxybenzoate, followed by hydrolysis of ester groups to carboxylic acid as described in Allcock, H. R. & Kwon, S. (1989) *Macromolecules* 22, 75-79, the teachings of which are incorporated
10 herein.

Example 3: Synthesis of poly[(carboxylatophenoxy) (chloro)phosphazene].

Poly[(carboxylatophenoxy) (chloro)phosphazene]
15 was prepared as follows.
Poly[di(chloro)phosphazene] (5.0 g, 0.0425 moles) was dissolved in 300 mL tetrahydrofuran (THF). The sodium salt of propyl p-hydroxybenzoate, prepared by reacting propyl hydroxybenzoate (30.6 g, 0.17
20 moles) with 60 % sodium hydride (6.12 g, 0.15 moles) in THF, was added dropwise to the dissolved polymer. After addition of the sodium salt, the reaction mixture was stirred at reflux for 2 days and monitored by ^{31}P NMR. The polymer was purified
25 by repeated precipitations into water, ethanol and hexane.

Poly[(propylhydroxybenzoate) (chloro)phosphazene] (2.0 g) was dissolved in 200 mL dry THF. 20 g potassium tert-butoxide was dissolved in 200 mL
30 THF. The basic solution was cooled to 0°C. Water (1 mL) was added to the butoxide/THF solution and stirred for 5 minutes. The polymer solution then was added dropwise to the aqueous base. The reaction mixture was warmed to room temperature and
35 stirred for 40 hours. After 40 hours, the reaction mixture was poured over an ice-water mixture and the THF was allowed to evaporate. The aqueous solution was then dialyzed against water for 2

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days. After dialysis was complete, the dialysate was acidified with HCl and the resultant white precipitate, poly[(carboxylatophenoxy)(chloro)phosphazene], was washed with water and filtered from the solution.

Example 4: Degradation of Phosphazene Polyelectrolytes

Degradation of phosphazene polyelectrolytes was studied *in vitro* at 37°C, in an air gravity incubator (Imperial II Incubator, Lab-Line Instruments, Inc.), with gentle agitation on a rotating shaker (ORBIT Shaker, Lab-Line Instruments, Inc., Melrose Park, Ill.) in vials containing a solution of 50 mg of polymer in 5 ml of 13 mM HEPES buffered saline solution (pH 7.4). The molecular weight of polyphosphazenes was determined by a Perkin-Elmer Series 10 liquid chromatograph with refractive index and a refractive index detector by using an Ultragel 2000 column (Waters Chromatography Division, Millipore Corporation, Cidra, Puerto Rico). 13 mM Hepes buffered saline solution (pH 7.4) was used as an eluant. Chromatograms were processed by GPC 5 and CHROM 2 software (Perkin-Elmer) to calculate the weight-average and number-average molecular weights using polyacrylic acid as a standard. The decline in polymer molecular weight over time is shown in Table 2.

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Table 2. **Degradation of**
Poly[(carboxylatophenoxy)
(glycinato)phosphazene].

| | Time days | Weight average molecular weight kDa | Number average molecular weight kDa |
|----|----------------------------|--|--|
| 5 | 0 | 132.0 | 70.0 |
| | 15 | 40.6 | 13.8 |
| | 60 | 6.3 | 1.5 |
| 10 | 180 | 6.0 | 0.9 |
| | 240 | 0.9 | 0.5 |

Example 5: Antibody Titers after Immunization
with Tetanus Toxoid in Various
Concentrations of a Polyphosphazene
Adjuvant.

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Antibody titers were determined in female BALB/c mice, age 7 to 8 weeks, that had been inoculated with tetanus toxoid admixed with a polyphosphazene adjuvant.

20

An immunogenic composition containing tetanus toxoid in polyphosphazene was prepared as follows. 100 mg of poly[di(carboxylatophenoxy)phosphazene] was dissolved in 1 ml Na_2CO_3 and 1 ml phosphate buffered saline (PBS), pH 7.2 was added. 1.4 ml tetanus toxoid (2.2 mg/ml or 1000 LF/ml, Connaught Laboratories, Inc., Swiftwater, PA) was added with 0.6 ml containing 0.025% Brij solution (10 μl of 10% Brij 58, Sigma Chemical Co., St. Louis, MO) to the polymer.

25

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Groups of five mice were immunized subcutaneously with a single dose of 25 μg tetanus toxoid admixed with dilutions containing 0.5% polyphosphazene, 0.05% polyphosphazene, or 0.005% polyphosphazene in dH_2O . A separate group of mice was immunized with a single subcutaneous dose of 25 μg of tetanus toxoid in complete Freund's adjuvant (SIGMA, St. Louis, MO). Blood samples were taken from the retroorbital sinus of CO_2 anaesthetized mice and analyzed by an ELISA immunoassay for anti-

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tetanus toxoid IgG.

As shown in Table 3 below, 25 μ g of tetanus
toxoid in 0.5% polyphosphazene gave anti-tetanus
specific serum IgG titers slightly higher than the
5 same dose in complete Freund's adjuvant. 25 μ g of
tetanus toxoid in 0.05% polyphosphazene gave titers
that were slightly lower than the same dose in
complete Freund's adjuvant. In all cases, the
titer of antibody against tetanus toxoid was
10 significantly higher when administered in
conjunction with polymer than when administered
alone.

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Table 3: Anti-tetanus titers in mice after administration of tetanus toxoid with a polyphosphazene adjuvant or Freund's adjuvant.

| Group | Treatment | Anti-tetanus toxoid ELISA titer (log 2) | | |
|------------|------------------------|---|------------|------------|
| | | Week 3 | Week 5 | Week 7 |
| 25 μ g | TT/0.5% PP | 65536(16) | 262144(18) | 524288(19) |
| 25 μ g | TT/0.05% PP | 16384(14) | 32768(15) | 32768(15) |
| 25 μ g | TT/0.005% PP | 4096(12) | 8192(13) | 32768(15) |
| 25 μ g | TT in Freund's | 16384(14) | 131072(17) | 262144(18) |
| 25 μ g | TT in H ₂ O | 1024(10) | 2048(11) | 2048(11) |

The letters "TT" are an abbreviation for tetanus toxoid whereas "pp" is an abbreviation for polyphosphazene.

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Example 6: Antibody Titers after Immunization with Various Concentrations of Tetanus Toxoid or Influenza Admixed with a Polyphosphazene Adjuvant.

5 Antibody titers were determined in female BALB/c mice, age 7 to 8 weeks, that had been inoculated with either influenza or tetanus toxoid admixed with the polyphosphazene adjuvant described in Example 5.

10 Two immunogenic compositions, one containing tetanus toxoid in polyphosphazene and the other containing influenza in polyphosphazene, were prepared as follows. 100 mg of
15 poly[di(carboxylatophenoxy) phosphazene] was dissolved in 1 ml Na_2CO_3 and 1 ml phosphate buffered saline (PBS), pH 7.6 was added. Subsequently,
20 tetanus toxoid (2.2 mg/ml) (Connaught Laboratories, Swiftwater, PA) or influenza (Influenza Branch, Center for Disease Control, Atlanta, GA) was
20 diluted 1:10 in water and the appropriate volume of each was admixed with either 0.1% or 0.05% polyphosphazene.

 Groups of three mice were immunized subcutaneously with a single dose of each
25 immunogenic composition. Blood samples were taken from the retroorbital sinus of CO_2 anaesthetized mice after 21 days after inoculation and analyzed by an ELISA immunoassay for anti-tetanus toxoid or anti-influenza IgG.

30 The following influenza immunoassay protocol was performed to determine the influenza titers: 96-well ELISA microtiter plates were coated with
 influenza cell lysates at 10 $\mu\text{g/ml}$ in carbonate buffer, pH 9.6, 100 μl per well and incubated 2
35 hours at 37°C. The plate was washed with 0.05% Tween 20/PBS (Sigma, St. Louis, MO) and 100 μl 2.5% bovine serum albumin/phosphate buffered saline (BSA/PBS) was added to each well as a blocking

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step. The plate was then incubated 1 hour at 37°C and washed with 0.05% Tween 20/PBS. 50 µl 1% BSA/PBS was added to all wells. Serum samples were diluted to 1:128 by adding 5 µl serum to 635 µl 1% BSA/PBS. 50 µl of the dilute serum sample to be assayed was added to the first well in a row, a 1:256 dilution. Both positive and negative controls were tested. Two-fold serial dilutions of serum sample were made by removing 50 µl from the first well in a row and adding the 50 µl with mixing to the second well; then removing 50 µl from the second well and adding it to the third well with mixing, and so on down the row, discarding 50 µl from the final or 12th well. The plates were then incubated 1 hour at 37°C and the plate washed with 0.05% Tween 20/PBS. To each well was added 100 µl of OPD solution (0.4 mg/ml solution of 0-phenylenediamine dihydrochloride (Sigma, St. Louis, MO) in 0.05 M phosphate-citrate buffer pH 5.0 (1 OPD tablet per 12.5 ml citrate buffer) containing 0.05% hydrogen peroxide (20.8 µl 30% H₂O₂ per 12.5 ml citrate buffer)). The color was allowed to develop for 30 minutes, then stopped by addition of 50 µl 2 M H₂SO₄/ well. The absorbance was read at OD₄₉₀, and the endpoint titer determined by finding the dilution of each serum sample that had an OD₄₉₀ greater than or equal to two times the OD₄₉₀ of the negative control at the same dilution.

As shown below in Tables 4 and 5, 25 mg of tetanus toxoid (TT) and 5 µg of influenza (flu), in combination with 0.1% polyphosphazene (PP), yielded serum IgG titers that were the same or higher than the same dose of antigen administered in complete Freund's adjuvant.

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Table 4: Antibody Titers After Administration of Tetanus Toxoid Admixed with a Polyphosphazene Adjuvant or Freund's adjuvant.

| <u>Treatment</u> | <u>Anti-Tetanus Toxoid Titer (log2)</u> |
|----------------------|---|
| 25 µg TT/0.1% PP | 16384 (14) |
| 5 µg TT/0.1% PP | 4096 (12) |
| 1 µg TT/0.1% PP | 2048 (11) |
| 0.2 µg TT/0.1% PP | 512 (9) |
| 25 µg TT/0.05% PP | 8192 (13) |
| 5 µg TT/0.05% PP | 4096 (12) |
| 1 µg TT/0.05% PP | 2048 (11) |
| 0.2 µg TT/0.05% PP | 512 (9) |
| 25 µg TT in water | 2048 (11) |
| 25 µg TT in Freund's | 16384 (14) |

Table 5: Antibody Titers After Administration of Influenza Admixed with a Polyphosphazene Adjuvant or Freund's adjuvant.

| <u>Treatment</u> | <u>Anti-Influenza Titer (log2)</u> |
|----------------------|------------------------------------|
| 5 µg flu/0.1% PP | 2048 (11) |
| 1 µg flu/0.1% PP | 4096 (12) |
| 0.2 µg flu/0.1% PP | < 256 (<8) |
| 0.04 µg flu/0.1% PP | < 256 (<8) |
| 5 µg flu/0.05% PP | 4096 (12) |
| 1 µg flu/0.05% PP | 1024 (10) |
| 0.2 µg flu/0.05% PP | < 256 (<8) |
| 0.04 µg flu/0.05% PP | < 256 (<8) |
| 5 µg flu in water | 256 (8) |
| 5 µg flu in Freund's | 512 (9) |

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**Example 7: Influenza Hemagglutination Inhibition
Titers after Immunization with
Influenza Admixed with a
Polyphosphazene Adjuvant.**

5 An influenza hemagglutination inhibition
antibody assay was performed with heat-inactivated
mouse serum that had been incubated for 30 minutes
with 10% chicken red blood cells (Spafas, Storrs,
CT) to remove non-specific inhibitors. Two-fold
10 dilutions of sera were added to a 96-well
microtiter plate and 8 hemagglutination (HA) units
of virus suspension in a equal volume were added to
each well and incubated at room temperature for 30
minutes. A 0.5% suspension of chicken red blood
15 cells was added to each well and incubated at room
temperature for 45-60 minutes. Hemagglutination
inhibition (HI) titers are expressed in Table 6
below as the reciprocal of the highest dilution
that completely inhibits hemagglutination of
20 erythrocytes.

As shown in Table 6, hemagglutination titers of
animals inoculated with flu in combination with 0.1
% polyphosphazene were high, while titers for flu
in combination with complete Freund's adjuvant were
25 negative.

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Table 6: Hemagglutination Inhibition Titers After Administration of Influenza Admixed with a Polyphosphazene Adjuvant or Freund's adjuvant.

| <u>Treatment Titers</u> | <u>Hemagglutination Inhibition</u> |
|---------------------------|------------------------------------|
| 5 μ g flu/0.1% PP | 160 |
| 1 μ g flu/0.1% PP | 320 |
| 0.2 μ g flu/0.1% PP | 40 |
| 0.04 μ g flu/0.1% PP | negative |
| 5 μ g flu/0.05% PP | 160 |
| 1 μ g flu/0.05% PP | 160 |
| 0.2 μ g flu/0.05% PP | negative |
| 0.04 μ g flu/0.05% PP | negative |
| 5 μ g flu in water | negative |
| 5 μ g flu in Freund's | negative |

Example 8: Antibody Titers after Immunization with Tetanus Toxoid or Influenza Admixed with Various Concentrations of Two Different Polyphosphazene Polymer Adjuvants.

5 100 mg of poly[di(carboxylatophenoxy)phosphazene] (Polymer 1) was dissolved in 1 ml Na_2CO_3 and 3 ml of PBS was added to the polymer solution. 100 mg of poly[(carboxylatophenoxy)-
10 (glycinato)phosphazene] (Polymer 2) was dissolved in the same solvents.

Antibody titers were determined in groups of female BALB/c mice, three mice per group, age 7 to 8 weeks, 21 days after inoculation with 5 μ g
15 influenza admixed with one of each of the polymers.

As shown below in Table 7, 5 μ g of flu in 0.1 % or 0.05 % of polymer 1 or polymer 2 give serum IgG titers that are higher than the same dosage of antigen in complete Freund's adjuvant.

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Table 7: Antibody Titers After Administration of Influenza Admixed with Poly[di(carboxylatophenoxy)phosphazene] (Polymer 1), Poly[(carboxylatophenoxy)-(glycinato)phosphazene] (Polymer 2) or Freund's adjuvant.

| <u>Treatment</u> | <u>Anti-Influenza Titer (log2)</u> |
|--------------------------|------------------------------------|
| 5 µg flu in water | <256 (<8) |
| 5 µg flu in Freund's | 256 (8) |
| 5 µg flu/0.1% Polymer 2 | 1024 (10) |
| 5 µg flu/0.05% Polymer 2 | 512 (9) |
| 5 µg flu/0.01% Polymer 1 | 4096 (12) |
| 5 µg flu/0.05% Polymer 1 | 4096 (12) |

Modifications and variations of the present invention, polymer adjuvants and methods of synthesis and use in vaccine compositions, will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the appended claims.

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We claim:

1. A polyphosphazene selected from the group consisting of poly[di(carboxylatophenoxy)-phosphazene-co-di(glycinato)phosphazene-co-(carboxylatophenoxy)(glycinato)phosphazene], poly[di(carboxylatophenoxy)phosphazene-co-di(chloro)phosphazene-co-(carboxylatophenoxy)(chloro)phosphazene], and poly[di(carboxylatophenoxy)phosphazene-co-di(amino acid)phosphazene-co-(carboxylatophenoxy)(amino acid)phosphazene].
2. The polymer of claim 1 further comprising a pharmaceutical carrier.
3. The polymer of claim 1 further comprising an antigen.
4. The polymer of claim 1 cross-linked with a multivalent cation.
5. The polymer of claim 4 wherein the multivalent cation is selected from the group consisting of calcium, copper, aluminum, magnesium, strontium, barium, tin, zinc, iron, poly(amino acid), poly(ethyleneimine), poly(vinylamine) and polysaccharides.
6. The polymer of claim 3 wherein the antigen is selected from the group consisting of a compound derived from a cell, bacteria, or virus particle, or portion thereof, wherein the compound is selected from the group consisting of proteins, peptides, polysaccharides, glycoproteins, glycolipids, nucleic acid, or combinations thereof.
7. The polymer of claim 3 wherein the phosphazene polyelectrolyte is covalently conjugated with the antigen.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/07710A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C08G79/02 A61K9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C08G A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
| A | EP,A,0 168 277 (MERCK & CO) 15 January 1986 ---- | |
| A | BIOMATERIALS, vol.13, no.8, 1992, GUILDFORD GB pages 511 - 520 J.H.L.CROMMEN ET ALL. 'Biodegradable polymers I. Synthesis of hydrolysis-sensitive poly[(organo)phosphazenes]' ----- | |
| A | BIOMATERIALS, vol.13, no.9, 1992, GUILDFORD GB pages 601 - 611 J.H.L.CROMMEN ET ALL. 'Biodegradable polymers II. Degradation characteristics of hydrolysis-sensitive poly[(organo)phosphazenes]' ----- | |

☐ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

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| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| EP-A-0168277 | 15-01-86 | US-A- 4639366 | 27-01-87 |
| | | CA-A- 1250073 | 14-02-89 |
| | | JP-A- 61009431 | 17-01-86 |
| | | US-A- 4765973 | 23-08-88 |
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